HELLER EHRMAN WHITE & MCAULIFFE LLP
Sheet 1 of 23
Title: COLLECTIONS OF BINDING PROTEINS AND TAGS
AND USES THEREOF FOR NESTED SORTING AND
HIGH THROUGHPUT SCREENING.
Applicant: Ault-Riche *et al.*
Serial No. 09/910,120 Filed: July 18, 2001
Our Docket No.: 25885-1751

Sorting by pools

Master

F1   OOOO●OOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOO   42

F2   OOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOO●OOOOOOOOO   42

F3   OOOOOOOOOOOOOOOOOO●OOOOOOOOOOOOOOOOOOOOOOOO   42

$F1_5/F2_{31}/F3_{16}$ $(42)(42)(42) = 74,088$

FIG. I

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Sorting by pools: Decreasing pool diversities

Master ●   Groups: 1
Diversity: 74,088

F1   OOOO●OOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOO 42

Groups: 42
Diversity: 1,764

F2   Groups: 42
Diversity: 42

F3   Groups: 42
Diversity: 1

$F1_5/F2_{31}/F3_{16}$

FIG. 2

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Sorting by pools: Screening large diversity libraries

Master

96-well plate

F1

microarray

F2

microarray

F3
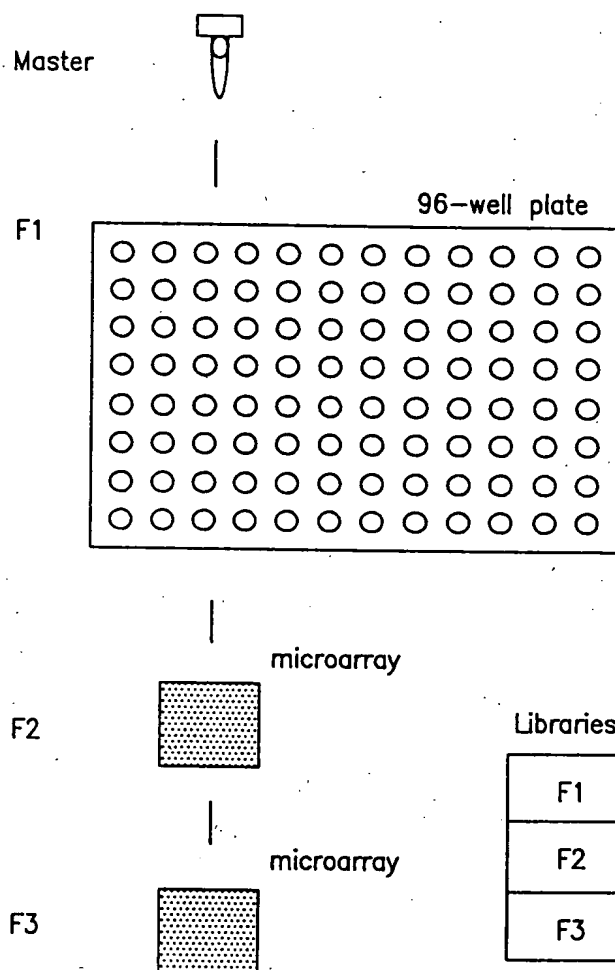
Libraries	Pools			
F1	100	1,000	100	1,000
F2	100	100	1,000	1,000
F3	100	100	1,000	1,000
Totals	10^6	10^7	10^8	10^9

FIG. 3

APPROVED | O.G. FIG.
BY | CLASS | SUBCLASS
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Searching a mutation library
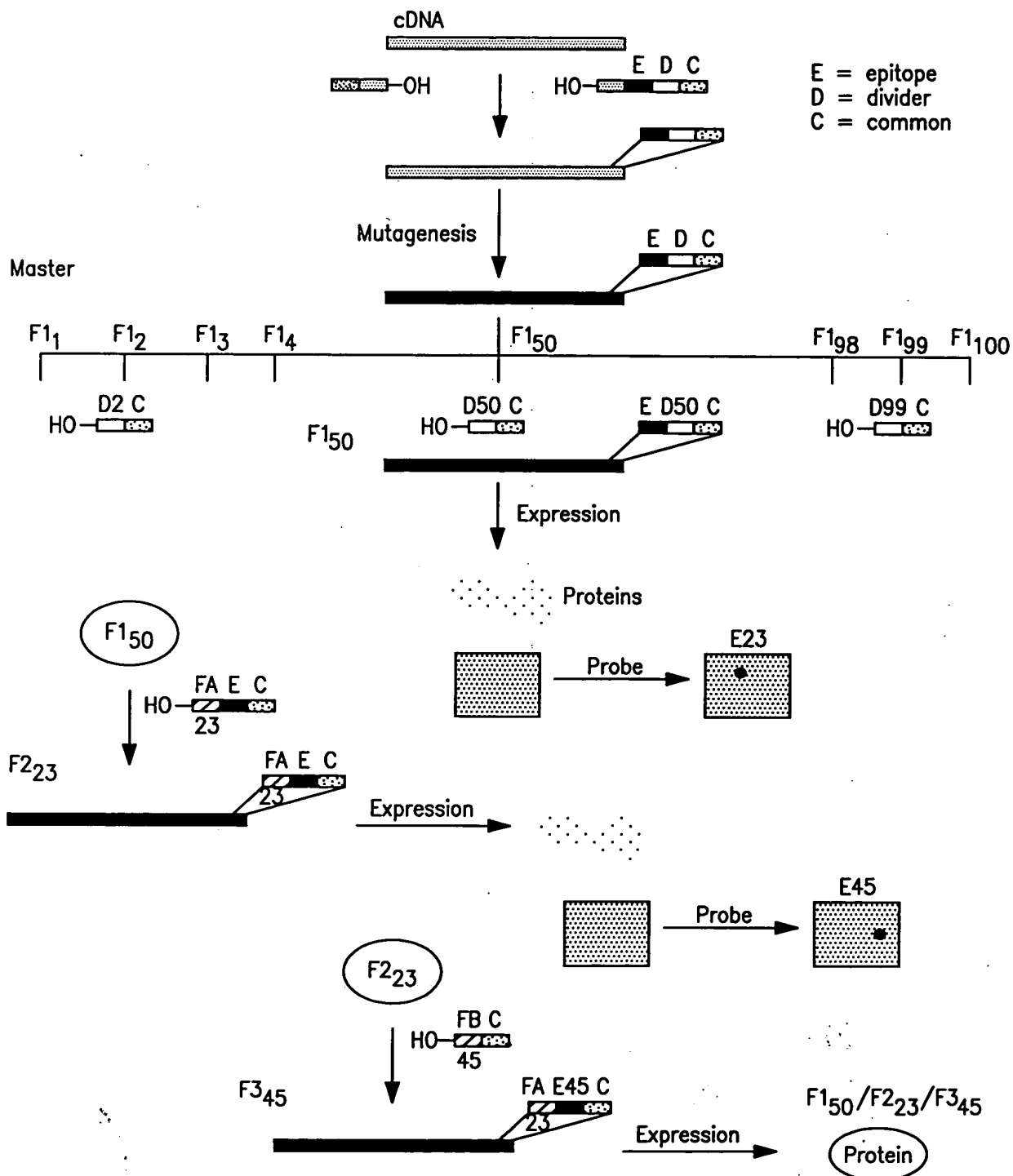
FIG. 4

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Making a recombinant antibody library

FIG. 5

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Applicant: Ault-Riche *et al.*
Serial No. 09/910,120 Filed: July 18, 2001
Our Docket No.: 25885-1751
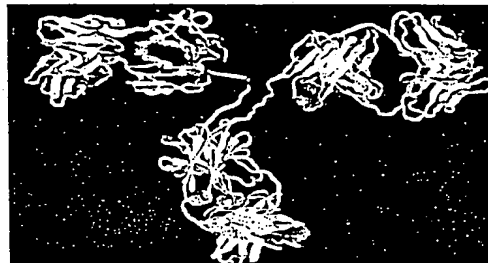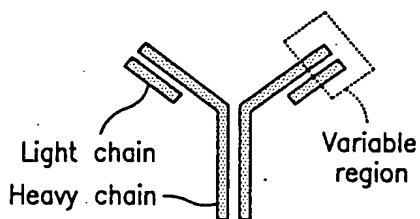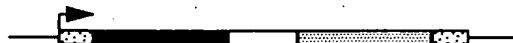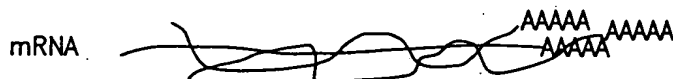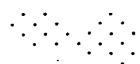
Creating the master antibody library: Primer incorporation

1. mRNA purification from spleen or PBLs

V_H V_L

2. cDNA synthesis

3. amplification

V_{HBACK} V_{LBACK}

V_{HFOR} V_{LFOR-C}

4. assembly

E   D   C

V_{HBACK} V_H Linker V_L

V_{LFOR-C}

V_H Primers

Oligo dT	HO–TTTTTTTT(T)$_n$ 3' 5'
V_{HBACK}	V_H back 5' –OH 3'
V_{HFOR}	J_H for OH– 3' 5'

V_L Primers

V_{LFOR}	J_{kappa} for E D C HO– 3' 5'
V_{LBACK}	V_{kappa} back 5' –OH 3'
V_{LFOR-C}	C HO– 3' · 5'

FIG. 6

Creating the master antibody library: Linker addition

1. mRNA purification from spleen or PBLs
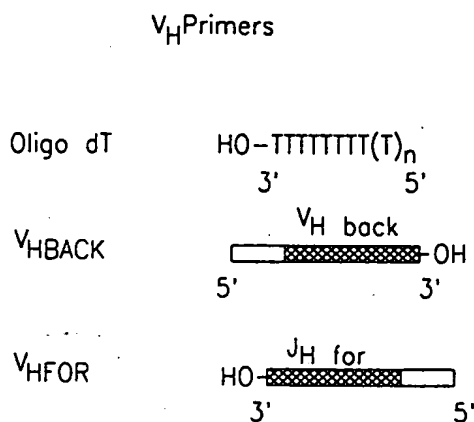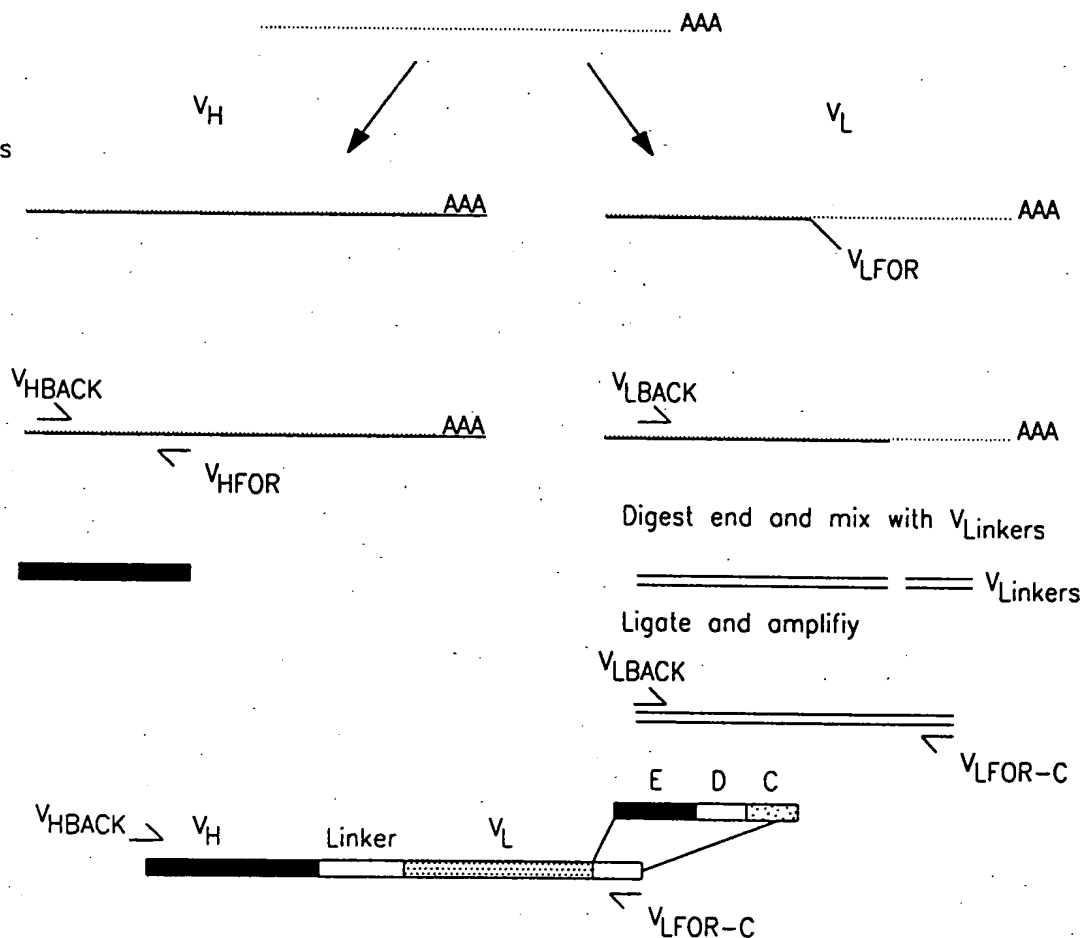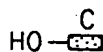
2. cDNA synthesis

3. amplification

Digest end and mix with $V_{Linkers}$

Ligate and amplifiy

4. assembly

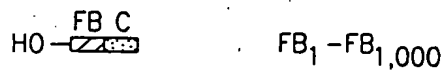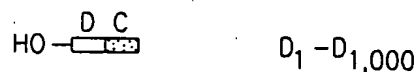

FIG. 7

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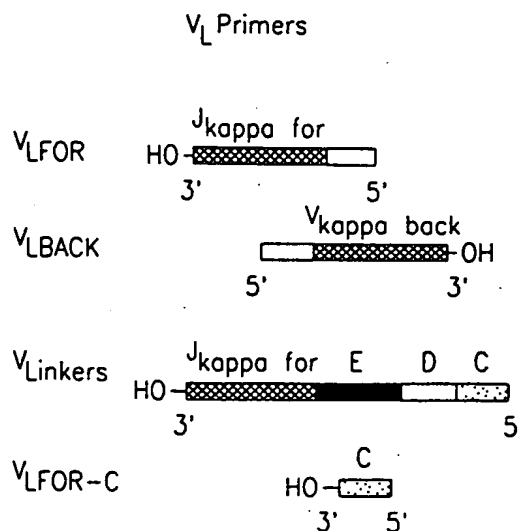
Searching a recombinant antibody library
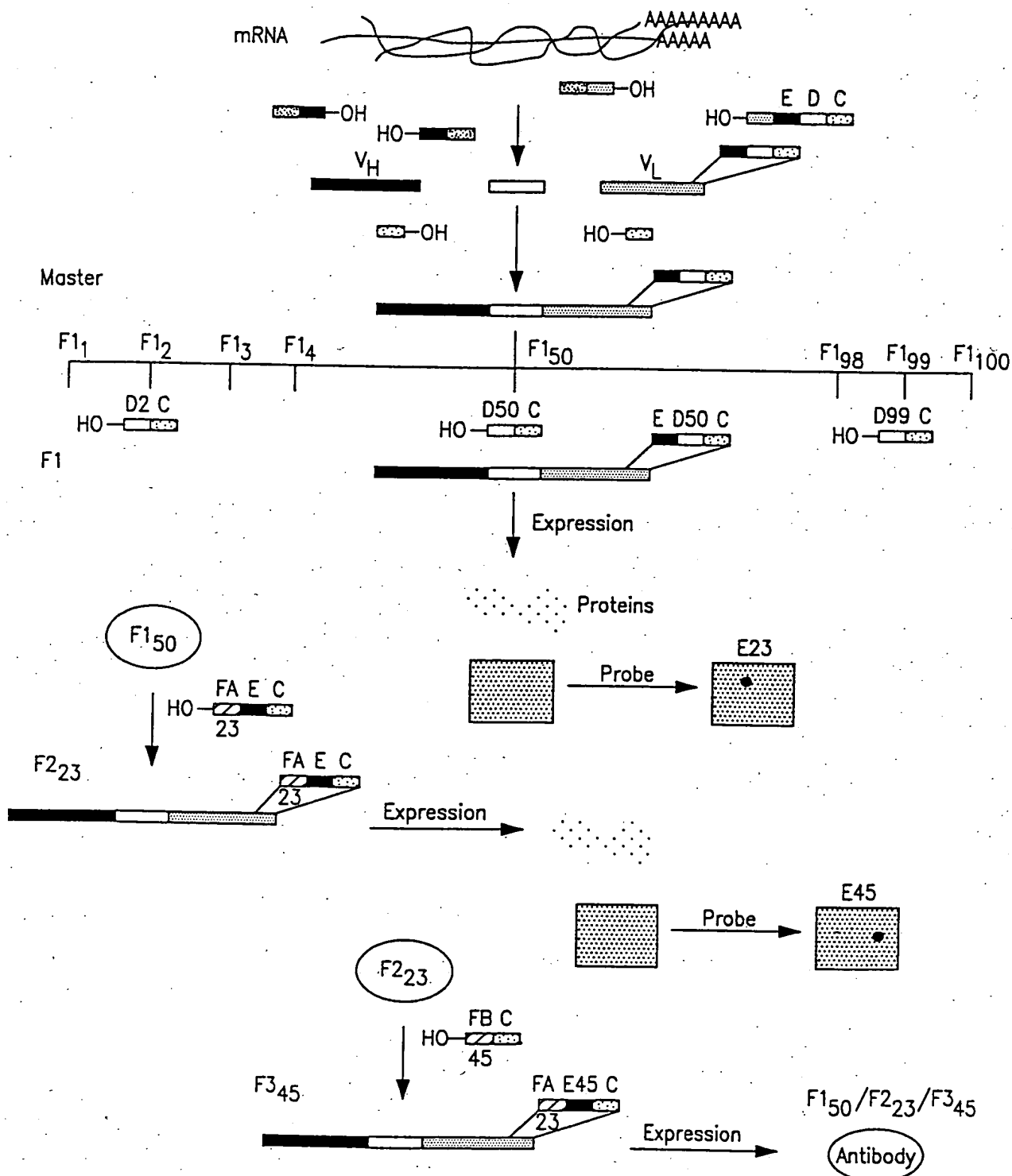
FIG. 8

Physical elements to include in the kits and combinations

- *Anti-tag Arrays*™

- Primer sets

  HO —■■□▣ E D C ⊗ $D_1 - D_{1,000}$

  HO —□▣ D C $D_1 - D_{1,000}$

  HO —▨■▣ FA E C $FA_1 - FA_{1,000}$

  HO —▨▣ FB C $FB_1 - FB_{1,000}$

  HO —▣ C

- Readers

- Software

FIG. 9

APPROVED	O.G. FIG.	
BY	CLASS	SUBCLASS
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Making the V$_{LFOR}$ primers: Solid phase synthesis
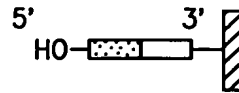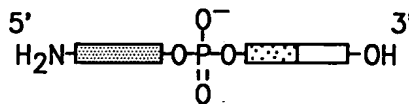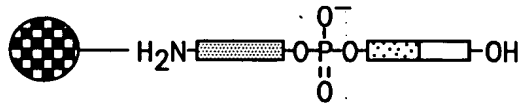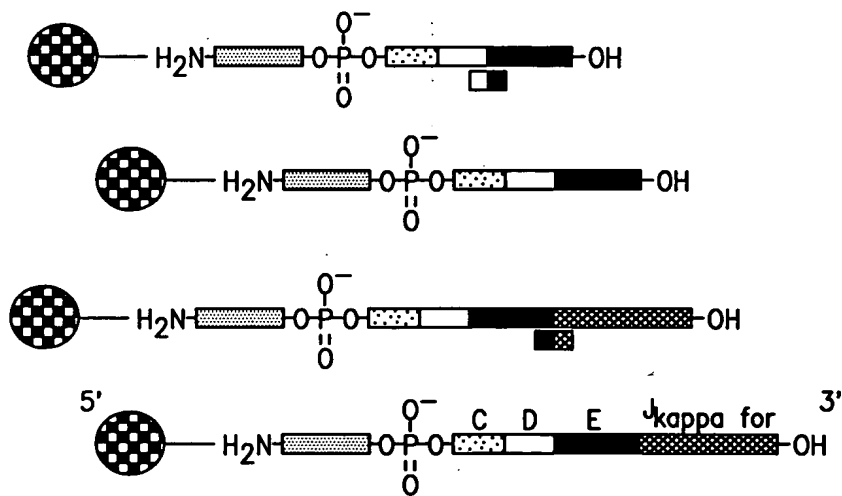
FIG. 10

APPROVED | O.6. . . C1.
BY | CLAS . . . . SS
DRAFTSMAN

Making the V_{LFOR} primers: Overlapping hybridiation

V_{LFOR}: HO — [J_{kappa} for][Epitope][D][Common]
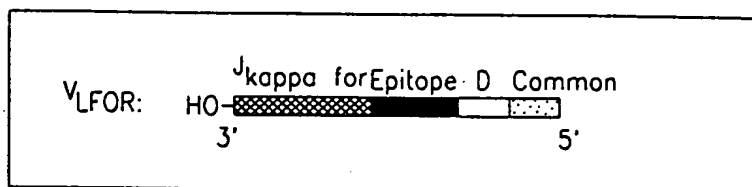
J_{kappa} for — 3'   5'

| J_{kappa} for | Epitope | D | Common |

- Synthesize 4,028 different oligos:
(26 for J_{kappa} for ; 2,000 for Epitope, 2,000 for D; 2 for Common

2. Assemble oligos for + and − strands of the different regions

OH
HO
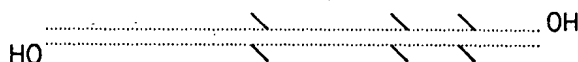
3. Ligase the assembled oligos

OH
HO

4. 1st strand synthesis with biotinylated primer

* _____ OH
HO
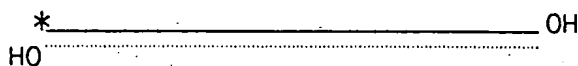
- 2nd strand synthesis with non−biotinylated primer
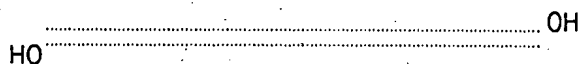
* _____ OH
HO
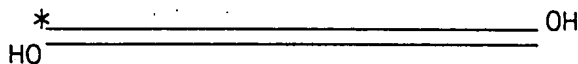
6. Bind to avidin coated magnetic beads and then denature

* _____ OH
HO
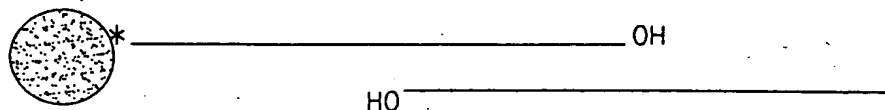
7. Purify non−biotinylated ssDNA

J_{kappa} for   Epitope   D   Common
HO

FIG. 11

APPROVED	O.G. FIG.	
BY	CLASS	SUBCLASS
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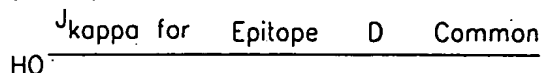
Building the collection of antibody/tag pairs: Hybridoma screening
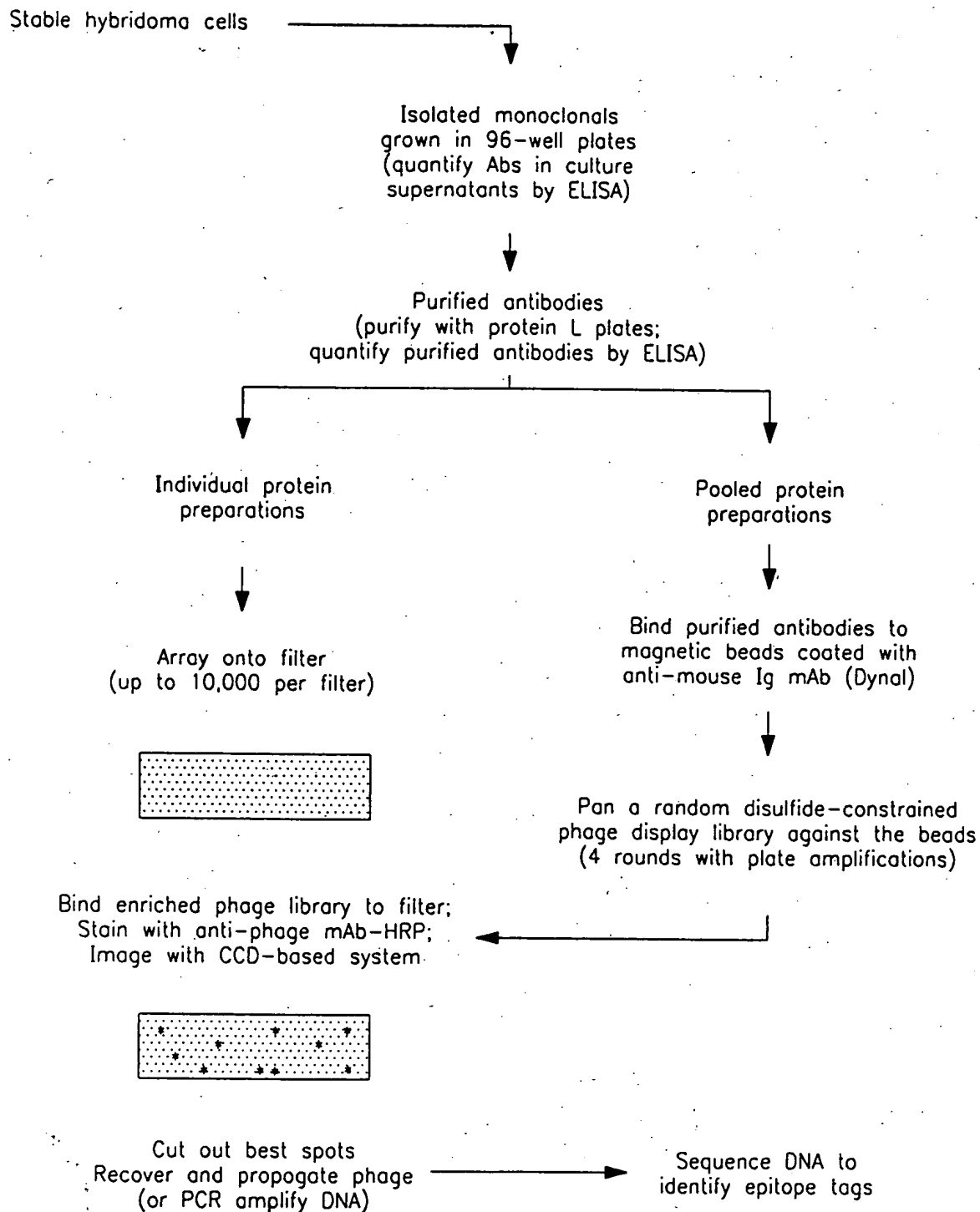
Stable hybridoma cells

Isolated monoclonals
grown in 96-well plates
(quantify Abs in culture
supernatants by ELISA)

Purified antibodies
(purify with protein L plates;
quantify purified antibodies by ELISA)

Individual protein
preparations

Pooled protein
preparations

Array onto filter
(up to 10,000 per filter)

Bind purified antibodies to
magnetic beads coated with
anti-mouse Ig mAb (Dynal)

Pan a random disulfide-constrained
phage display library against the beads
(4 rounds with plate amplifications)

Bind enriched phage library to filter;
Stain with anti-phage mAb-HRP;
Image with CCD-based system

Cut out best spots
Recover and propogate phage
(or PCR amplify DNA)

Sequence DNA to
identify epitope tags

FIG. 12

APPROVED	O.G. FIG.	
BY	CLASS	SUBCLASS
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<u>Table 3</u> Primers for PCR Amplification of Human Antibody Variable Regions (V genes)

1. V gene primary PCR

A. . Human VH back primers (sense)

HuVH1aBACK	5'-CAG GTG CAG CTG GTG CAG TCT GG-3'
HuVH2aBACK	.5'-CAG GTC AAC TTA AGG GAG TCT GG-3'
HuVH3aBACK	5'-GAG GTG CAC CTG GTG GAG TCT GG-3'
HuVH4aBACK	5'-CAG GTG CAG CTG CAG GAG TCG GG-3'
HuVH5aBACK	5'-GAG GTG CAG CTG TTG CAG TCT GC-3'
HuVH6aBACK	5'-CAG GTA CAG CTG CAG CAG TCA GG-3'

B. Human JH forward primers (anti-sense)

HuJH1-2FOR	5'-TGA GGA GAC GGT GAC CAG GGT GCC-3'
HuJH3FOR	5'-TGA AGA GAC GGT GAC CAT TGT CCC-3'
HuJH4-5FOR	5'-TGA GGA GAC GGT GAC CAG GGT TCC-3'
HuJH6FOR	5'-TGA GGA GAC GGT GAC CGT GGT·CCC-3'

C. Human V kappa back primers (sense)

HuVk1aBACK	5'-GAC ATC CAG ATG ACC CAG TCT CC-3'
HuVk2aBACK	5'-GAT GTT GTG ATG ACT CAG TCT CC-3'
HuVk3aBACK	5'-GAA ATT GTG TTG ACG CAG TCT CC-3'
HuVk4aBACK	5'-GAC ATC GTG ATG ACC CAG TCT CC-3'
HuVk5aBACK	5'-GAA ACG ACA CTC ACG CAG TCT CC-3'
HuVk6aBACK	5'-GAA ATT GTG CTG ACT CAG.TCT CC-3'

C. Human V lambda back primers (sense)

HuVλ1BACK	5'-CAG TCT GTG TTG ACG CAG CCG CC-3'
HuVλ2BACK	5'-CAG.TCT GCC CTG ACT CAG CCT GC-3'
HuVλ3aBACK	5'-TCC TAT GTG CTG ACT CAG CCA CC-3'
HuVλ3bBACK	5'-TCT TCT GAG CTG ACT CAG GAC CC-3'
HuVλ4BACK	5'-CAC GTT ATA CTG ACT CAA CCG CC-3'
HuVλ5BACK	5'-CAG GCT GTG CTC ACT CAG CCG TC-3'
HuVλ6BACK	5'-AAT TTT ATG CTG ACT CAG CCC CA-3'

D. Human J kappa forward primers (anti-sense)

HuJk1FOR	5'-ACG TTT GAT TTC CAC CTT GGT CCC-3'
HuJk2FOR	5'-ACG TTT GAT CTC CAG CTT GGT CCC-3'
HuJk3FOR	5'-ACG TTT GAT ATC CAC TTT GGT CCC-3'
HuJk4FOR	5'-ACG TTT GAT CTC CAC CTT GGT CCC-3'
HuJk5FOR	.5'-ACG TTT AAT CTC CAG TCG TGT CCC-3'.

D. Human J. lambda forward primers (anti-sense)

HuJλ1FOR	5'-ACC TAG GAC GGT GAC CTT GGT CCC-3'.
HuJλ2-3FOR	5'-ACC TAG GAC GGT CAG CTT GGT CCC-3'
HuJλ4-5FOR.	5'-ACC TAA AAC GGT GAG CTG GGT CCC-3'

FIG. 13A

APPROVED	O.G. FIG.
BY	CLASS · SUBCLASS
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2. Linker fragment PCR

F. Reverse JH for scFv linker (sense)

```
                        ——FR4 heavy ———————————┤ ├—linker
RHuJH1-2      5'-GC ACC CTG GTC ACC GTC TCC TCA GGT GG-3'
RHuJH3        5'-GG ACA ATG GTC ACC GTC TCT TCA GGT GG-3'
RHuJH4-5      5'-GA ACC CTG GTC ACC GTC TCC TCA GGT GG-3'
RHuJH6        5'-GG ACC ACG GTC ACC GTC TCC TCA GGT GG-3'
```

F. Reverse Vk for scFv linker (anti-sense)

```
                        ——FR1 light———————————┤ ├——linker
RHuVk1aBACKFv  5'-GG AGA CTG GGT CAT CTG GAT GTC CGA TCC GCC-3'
RHuVk2aBACKFv  5'-GG AGA CTG AGT CAT CAC AAC ATC CGA TCC GCC-3'
RHuVk3aBACKFv  5'-GG AGA CTG AGT CAA CAC AAT TTC CGA TCC GCC-3'
RHuVk4aBACKFv  5'-GG AGA CTG GGT CAT CAC GAT GTC CGA TCC GCC-3'
RHuVk5aBACKFv  5'-GG AGA CTG CGT GAG TGT CGT TTC CGA TCC GCC-3'
RHuVk6aBACKFv  5'-GG AGA CTG AGT CAG CAC AAT TTC CGA TCC GCC-3'
```

F. Reverse Vλ for scFv linker (anti-sense)

```
                        ————FR1 light——————————┤ ├————linker ———————
RHuVλBACK1Fv   5'-GG CGG CTG CGT CAA CAC AGA CTG CGA TCC GCC ACC GCC AGA G-3'
RHuVλBACK2Fv   5'-GC AGG CTG AGT CAG AGC AGA CTG CGA TCC GCC ACC GCC AGA G-3'
RHuVλBACK3aFv  5'-GG TGG CTG AGT CAG CAC ATA GGA CGA TCC GCC ACC GCC AGA G-3'
RHuVλBACK3bFv  5'-GG GTC CTG AGT CAG CTC AGA AGA CGA TCC GCC ACC GCC AGA G-3'
RHuVλBACK4Fv   5'-GC CGG TTG AGT CAG TAT AAC GTG CGA TCC GCC ACC GCC AGA G-3'
RHuVλBACK5Fv   5'-GA CGG CTG AGT CAG CAC AGA CTG CGA TCC GCC ACC GCC AGA G-3'
RhuVλBACK6Fv   5'-TG GGG CTG AGT CAG CAT AAA ATT CGA TCC GCC ACC GCC AGA G-3'
```

3. Pull-through primers for introduction of restriction sites*

G. Human VH back (Sfi)primers (sense)

```
HuVH1aBACKSfi                                       ┤——FR1 heavy ———————————
5'-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC CAG GTG CAG CTG GTG CAG TCT GG-3'
HuVH2aBACKSfi
5'-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC CAG GTC AAC TTA AGG GAG TCT GG-3'
HuVH3aBACKSfi
5'-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC GAG GTG CAG CTG GTG GAG TCT GG-3'
HuVH4aBACKSfi
5'-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC CAG GTG CAG CTG CAG GAG TCG GG-3'
HuVH5uBACKSfi
5'-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC CAG GTG CAG CTG TTG CAG TCT GC-3'
HuVH6aBACKSfi
5'-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC CAG GTA CAG CTG CAG CAG TCA GG-3'
```

H. Human J kappa forward (Not) primers (anti-sense)

```
HuJk1FORNot                          ┤——FR4 light ———————————
5'-GAG TCA TTC TCG ACT TGC GGC CGC ACG TTT GAT TTC CAC CTT GGT CCC-3'
HuJk2FORNot
5'-GAG TCA TTC TCG ACT TGC GGC CGC ACG TTT GAT CTC CAG CTT GGT CCC-3'
```
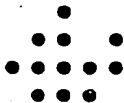
H. Human J kappa forward (Not) primers (anti-sense)(continued)

```
HuJk3FORNot                          ┤——FR4 light———————————
5'-GAG TCA TTC TCG ACT TGC GGC CGC ACG TTT GAT ATC CAC TTT GGT CCC-3'
HuJk4FORNot
5'-GAG TCA TTC TCG ACT TGC GGC CGC ACG TTT GAT CTC CAC CTT GGT CCC-3'
HuJk5FORNot
5'-GAG TCA TTC TCG ACT TGC GGC CGC ACG TTT AAT CTC CAG TCG TGT CCC-3'
```

H. Human J lambda forward (Not) primers (anti-sense)

```
HuJl1FORNOT                                    FR4 light
5'-GAG TCA TTC TCG ACT TGC GGC CGC ACC TAG GAC GGT GAC CTT GGT CCC-3'
HuJl2-3FORNOT
5'-GAG TCA TTC TCG ACT TGC GGC CGC ACC TAG GAC GGT CAG CTT GGT CCC-3'
HuJl4-5FORNOT
5'-GAG TCA TTC TCG ACT TGC GGC CGC ACC TAA AAC GGT GAG CTG GGT CCC-3'
```

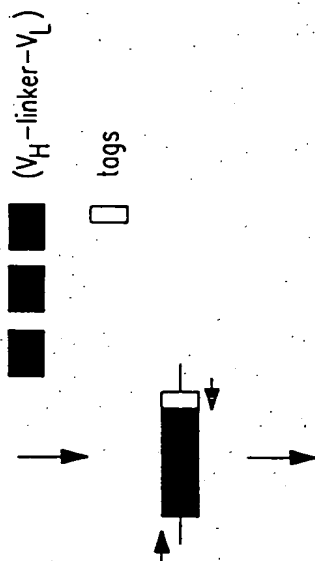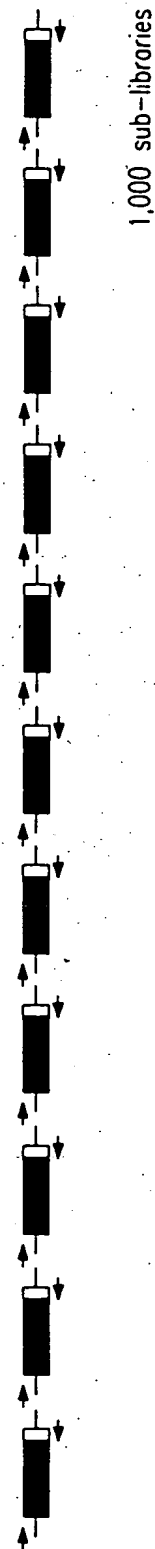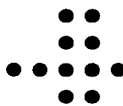*Recognition site for restriction enzyme is underlined.

FIG. 13B

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step I

Tag and assemble immunoglobulin genes

$(V_H-linker-V_L)$

tags

Create 1,000 sub-libraries by separate PCR amplification
reactions using tag-specific PCR primers

1,000 sub-libraries

FIG. 14A

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Applicant: Ault-Riche et al.
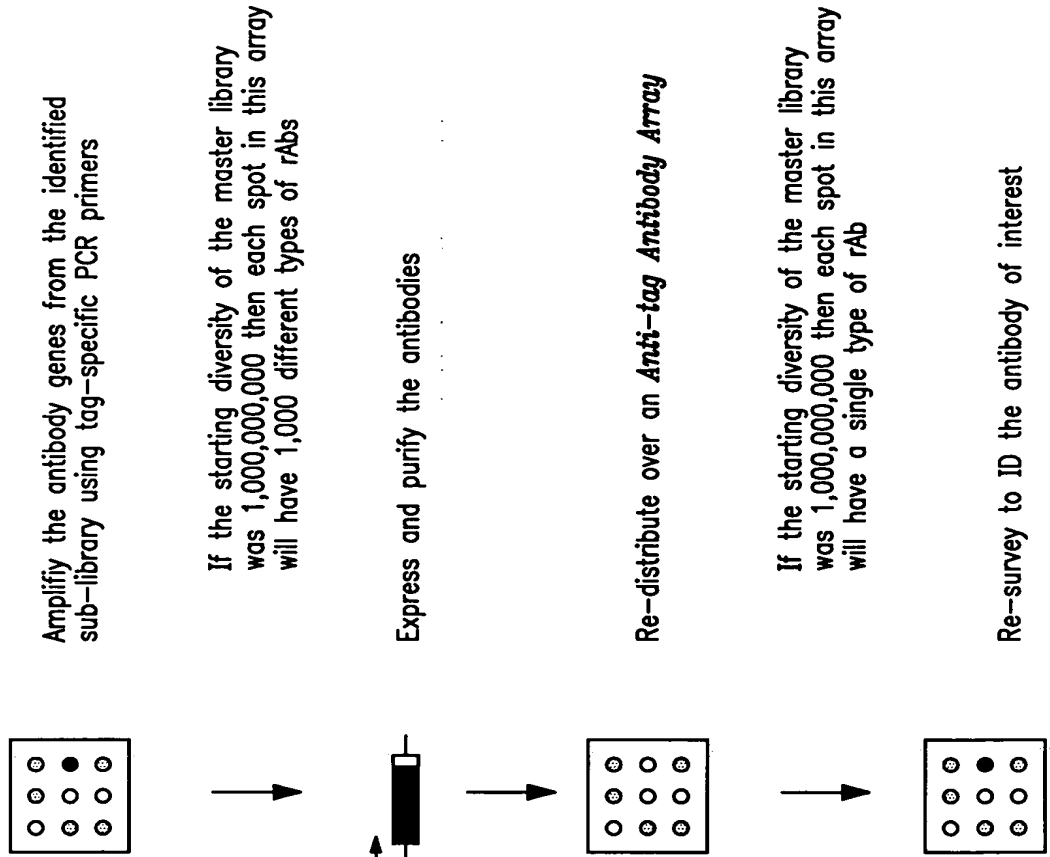Serial No. 09/910,120 Filed: July 18, 2001
Our Docket No.: 25885-1751

APPROVED | O.G. FIG.
·BY | CLASS | SUBCLASS
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step II

express antibodies from a sub-library and
bathe over an *Anti-tag Antibody Array*

bathe array with tagged antigen

Anti-tag
antibodies

Anti-tag antibody
with captured and
tagged antibodies
from a sub-library
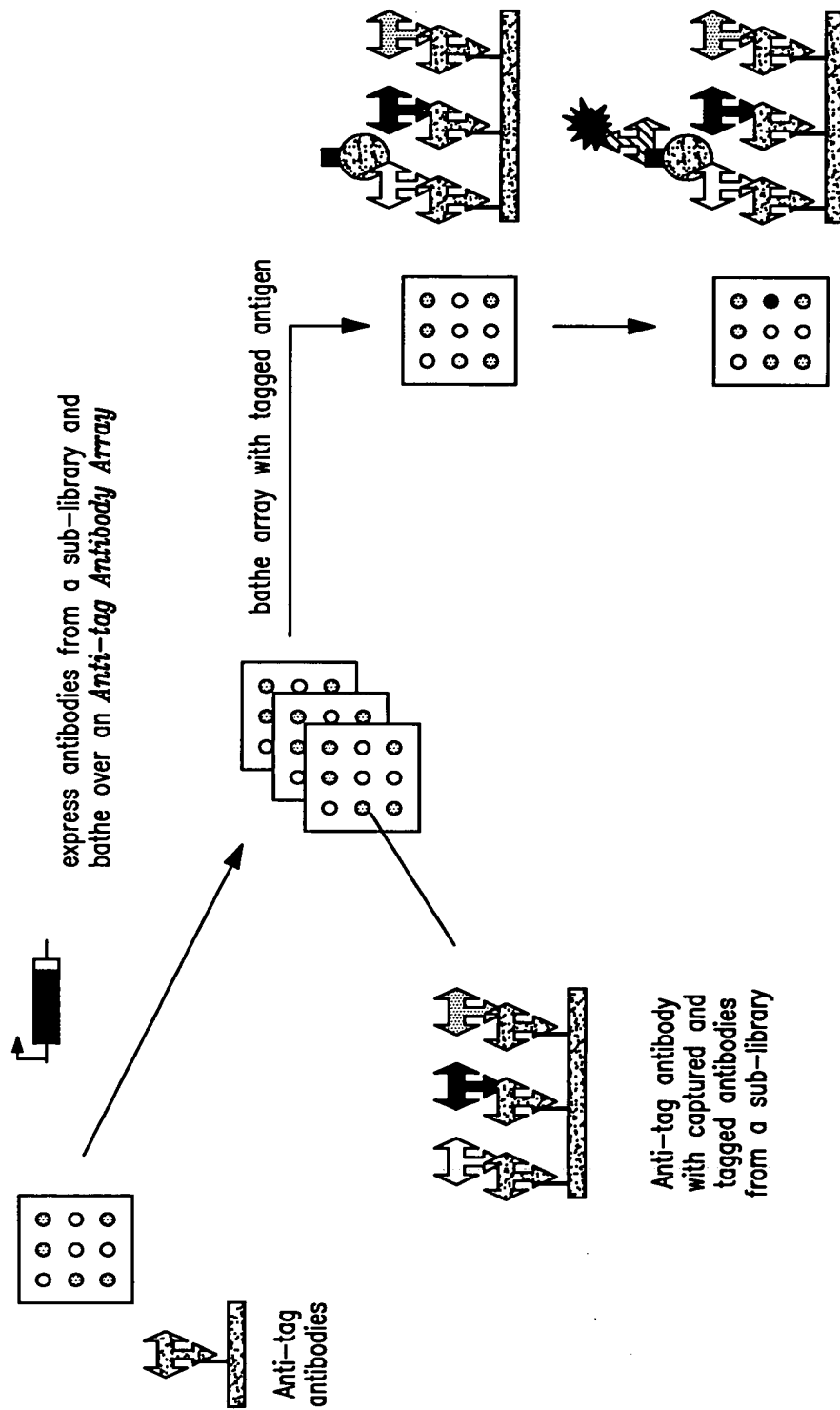
ID spot containing the antigen
with a labeled developing Ab

FIG. 14B

APPROVED	O.G. FIG.	·
BY	CLASS	SUBCLASS
DRAFTSMAN		

FIG. 14C

step III

Amplifiy the antibody genes from the identified
sub-library using tag-specific PCR primers

If the starting diversity of the master library
was 1,000,000,000 then each spot in this array
will have 1,000 different types of rAbs

Express and purify the antibodies

Re-distribute over an *Anti-tag Antibody Array*

If the starting diversity of the master library
was 1,000,000,000 then each spot in this array
will have a single type of rAb

Re-survey to ID the antibody of interest

FIG. 14D

summary

master library

1,000 sub-libraries
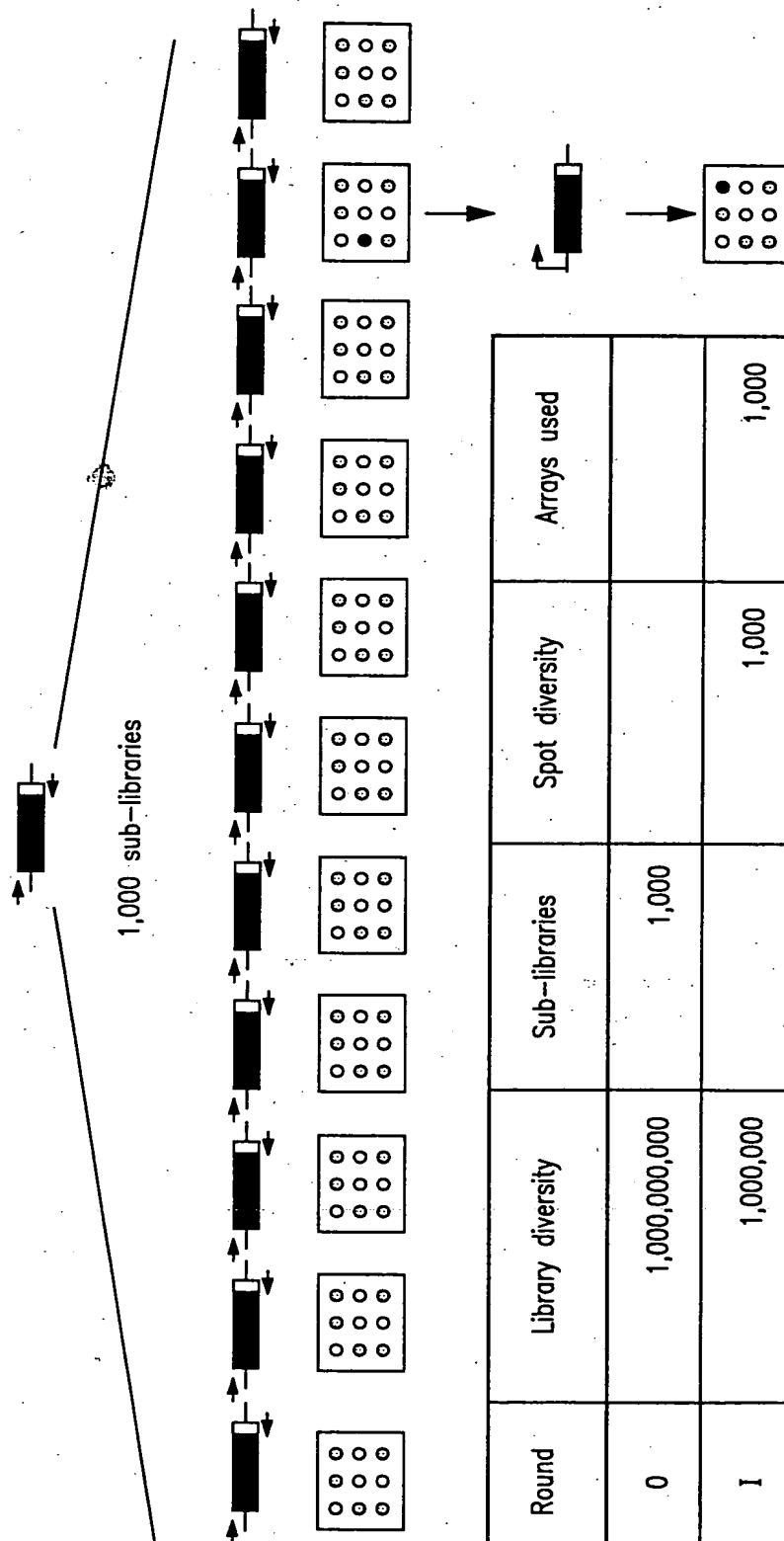
Round	Library diversity	Sub-libraries	Spot diversity	Arrays used
0	1,000,000,000	1,000		
I	1,000,000		1,000	1,000
II	1,000		1	1

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FIG. 15

Modification searches
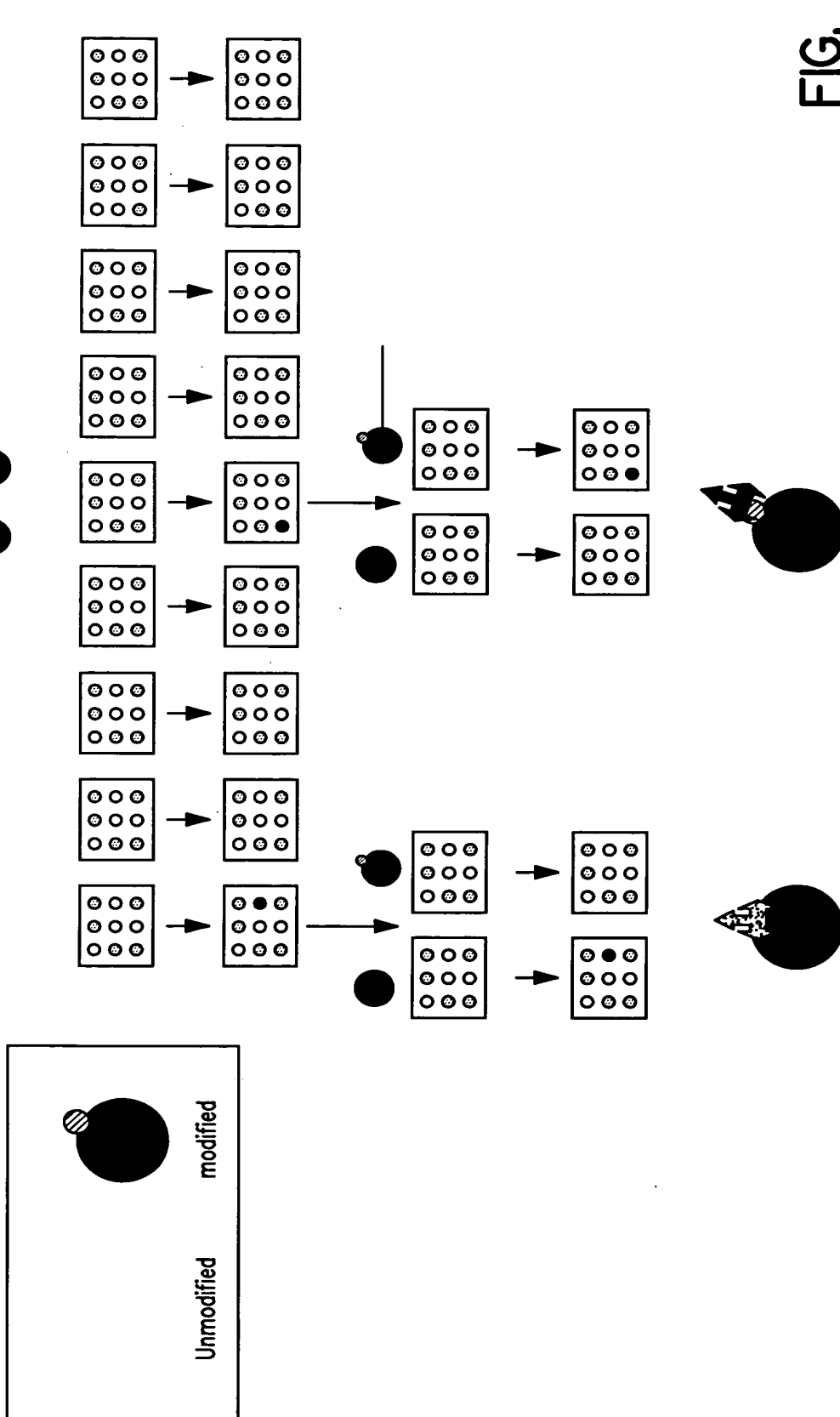
Unmodified   modified

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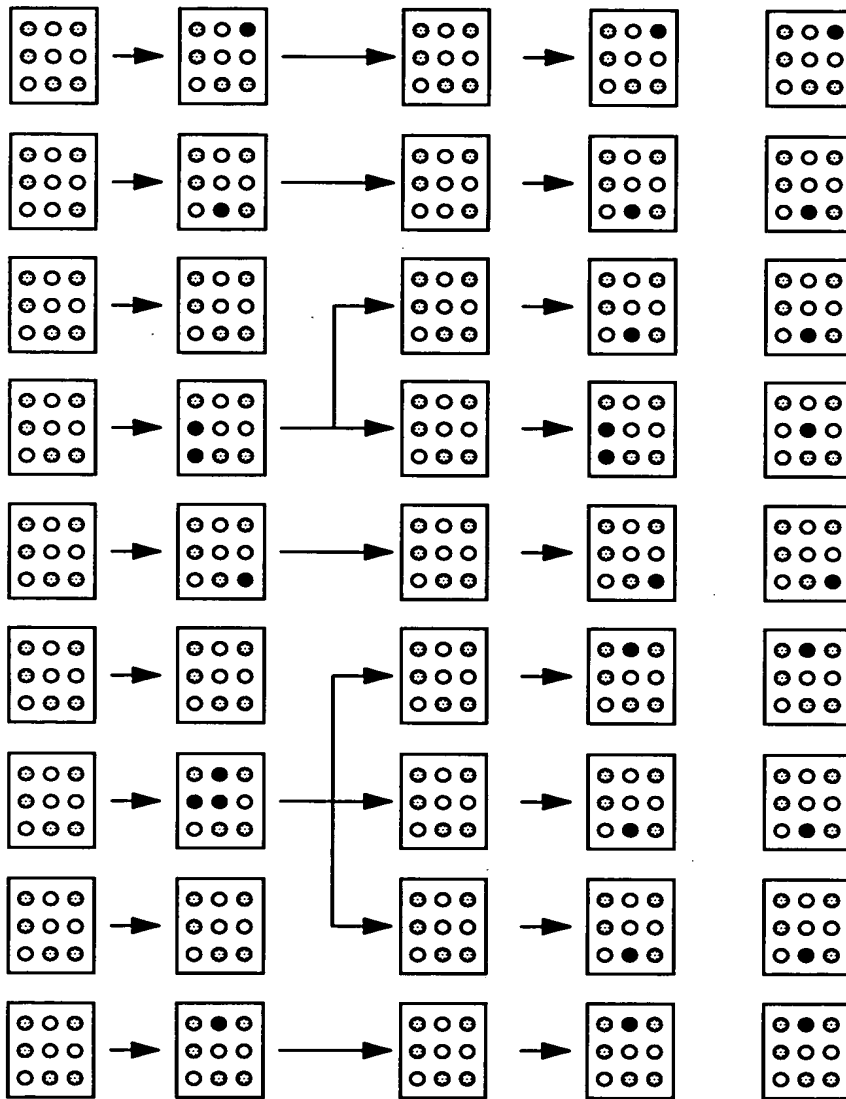Serial No. 09/910,120 Filed: July 18, 2001
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APPROVED	O.G. FIG.	
BY	CLASS	SUBCLASS
DRAFTSMAN		
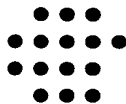
FIG. 16

Simultaneous searches

Round Arrays Bait Probe

I   1,000 Abs Ags

II   1,000 Abs Ags

III   1,000 Ags Abs
      ─────
      3,000

3 Arrays per Ag

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APPROVED	O.G. FIG.	
BY	CLASS	SUBCLASS
DRAFTSMAN		

Protein interaction mapping

Natural gene(s)

Error-prone PCR or Gene Shuffling

Mutated genes

• tag the genes to be mutated

• mutate genes and create sub-libraries

• distribute mutants over arrays

• probe the arrays with labeled substrates

Spots can contain mixtures of enzymes for detection or pathway engineering

FIG. 17

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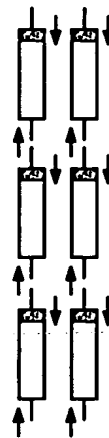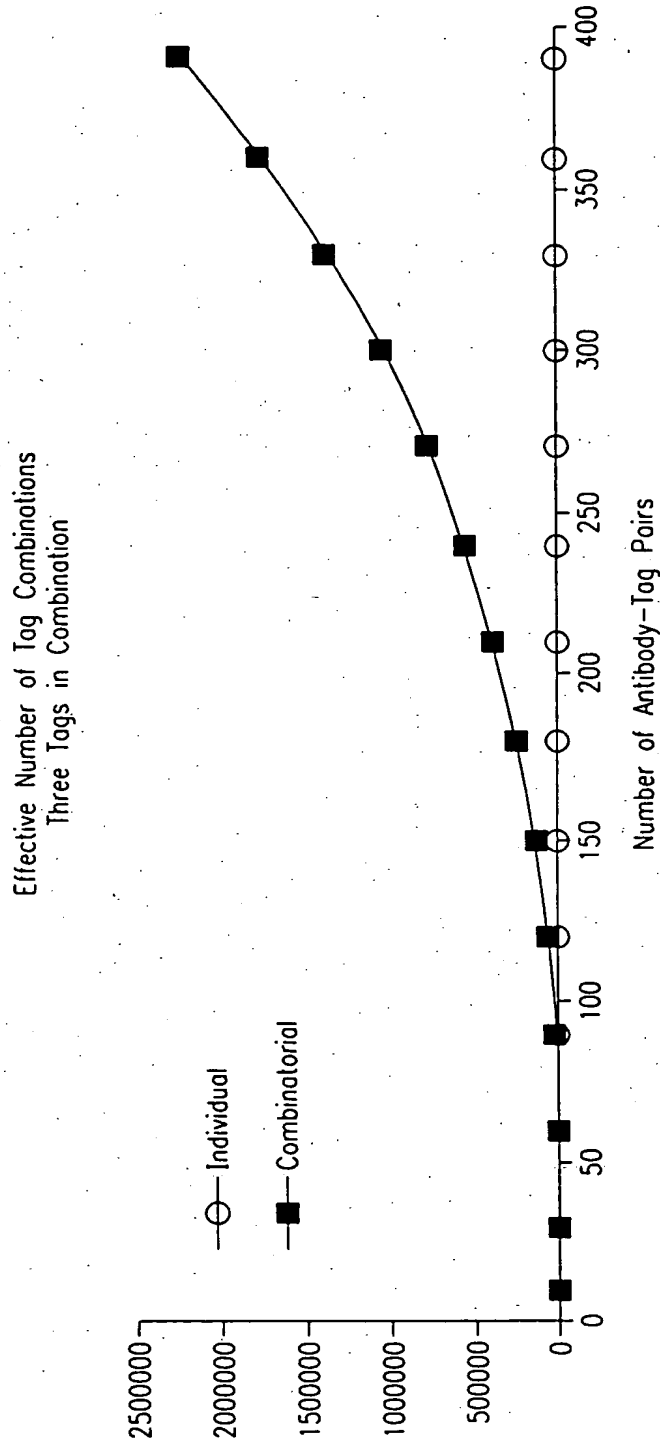Our Docket No.: 25885-1751
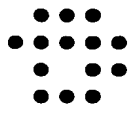
Protein interaction mapping

cDNA library

• Human tissue

• pathogen

• yeast

Generate a tagged cDNA library

Create sub-libraries by PCR

Distribute onto arrays
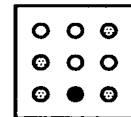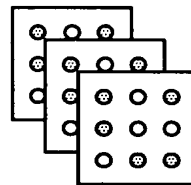
Probe arrays with one
or several labeled proteins,
peptides, or drugs

FIG. 18

Effective Number of Tag Combinations
Three Tags in Combination

FIG. 19